

7.88 Lecture Notes - 10
7.24/7.88J/5.48J
The Protein Folding Problem

Among non S-S proteins, one of those studied most intensively has been cytochrome c.

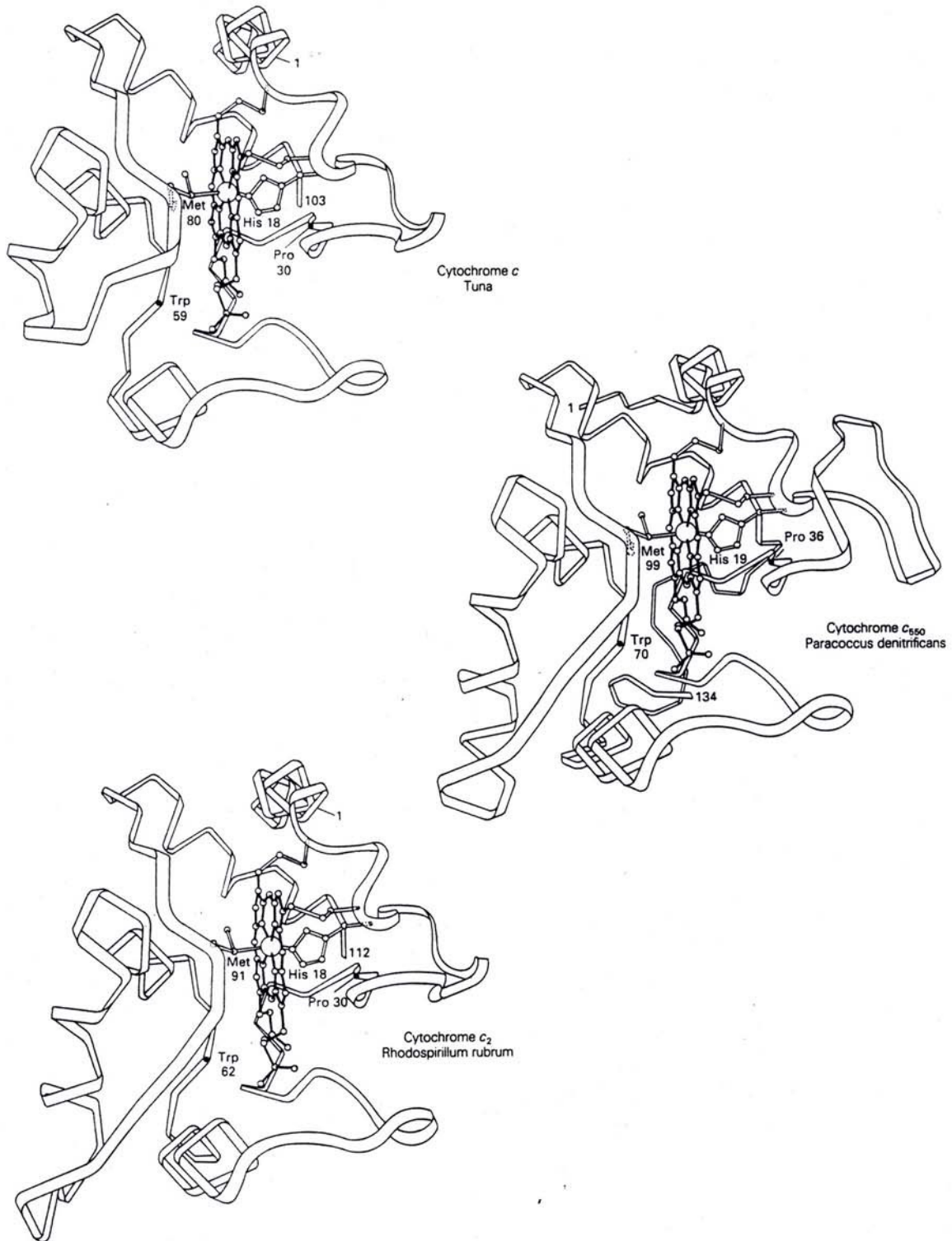
- **Cytochrome c structure**
- **Equilibrium unfolding/refolding and kinetic refolding studies**
- **Hydrogen/Deuterium Exchange**
- **Roder and Elove's experiments to characterize the early intermediate**

A. Cytochrome c - Central protein in electron transport pathway

Highly conserved through animal and plant kingdoms

Within mitochondrial membrane

Transparency of 3 species:



Courtesy of Annual Reviews. Figure 6.31: The similar folded conformations of distantly related cytochromes c.: Salemme FR. "Structure and function of cytochromes c." *Annu Rev Biochem.* 1977;46:299-329.

Figure 10.0: The Similar Folded Conformations of Distantly Related Cytochromes c (from Salemme, F. R. "Structure and function of cytochromes c." *Annu Rev Biochem* 46 (1977): 299-329.)

- Receives electron from cytochrome a/cytochrome c1 complex = cytochrome reductase;
- Passes it to cytochrome a/a3 = cytochrome oxidase;
- Alternates between oxidized (Fe+3) and reduced (Fe+2) states;

Structure: Reduced state:

- 104 amino acids, basic (17 lysines)
- No beta sheet; two well formed alpha helices: 1>11; 89>101
- Rest of chain is kind of wrapped around heme group
- Heme covalently linked to cysteine 14 and cysteine 17.
- In native state heme is liganded by Histidine 18 and Methionine 80.

In hemoglobin the Fe heme complex binds oxygen; in cytochrome it binds an electron, because the 6th coordinating site of the Iron that would bind O₂ is occupied by a methionine side chain.

Yeast has two cytochrome c's. called iso 1 and iso 2 , which differ at several places in sequence.

Iso-2 lacks free cysteine thiols, so not plagued by adventitious S-S bonds.

Exploration of Protein Database Structure:

B. Refolding of cytochrome c

Nall, Barry, and Terry Landers. "Guanidine HCl Induced Unfolding of Yeast Iso-2 Cytochrome c." *Biochemistry* 20 (1981): 5403-5411.

Equilibrium denaturation as function of GuHCl concentration;

Follow two uv absorption: A₂₈₇ (tryp/tyr) and A₄₁₈ = heme group.

Coincident with D_{1/2} around 1 Molar

Axes: Extinction coefficient vs. GdnHCL (pH 7.2, 20°C)

Looks two state, and seems to be concerted, aromatics exposed in same curve as heme.

They calculate Gibbs Free energy of 3.1kcal/mole, 20°C, extrapolated to 0 denaturant concentration.

Now lets carry out kinetic experiment;

Optical signal on left; 2 seconds/division on horizontal axis

Clearly two kinetic phases; fast phase, all done by 5 seconds, then slower phase time constant about 100 seconds

Similar kinetic phases regardless of which signal monitor, so not simply difference in heme relationship and overall conformation;

So indicates the **presence of some kinetic intermediate** in the folding pathway:

his18 ring remains ligated to heme iron under denaturing conditions.

Problem: How to determine structure; can't isolate it; transient intermediate

Methods for exploring conformation of transient folding intermediates:
X-ray diffraction?

Not presently possible...

C. Hydrogen Exchange with solvent

Hydrogen Exchange with solvent: (K. Linderstrom-Lang, Walter Englander U of P)

Detect exchange by incubating in tritiated water or deuterated water - substitution of tritium or deuterium for protons:

Exchange Rates for Hydrogens in Amino Acids:

- H atoms on C's: barely detectable
- H atoms on O, N and S: reasonably fast,
- Amide exchange, most valuable.

This is catalyzed by **both acid and base**, as shown for a set of model compounds. As a result enormous change of rate with Ph, but two branches

- Increases ten fold on either side for each unit of Ph
- Also Temperature dependent: three fold change for each 10°C increase in T. (Corresponds to activation of 17-20 kcal/mole)

Two mechanisms:

- **protonation of peptide C = O oxygen**, followed by transient loss of adjacent amide -NH hydrogen and then its replacement from solvent; during deprotonation.
- **Abstraction of amide Hydrogen directly** by OH⁻ hydroxide ion or H₂O, followed by replacement with another atom from solvent.

Thus the **Rates** of the reactions depend upon

- pH of solvent and
- tendency of groups to ionize

Backbone amide hydrogen: minimum at Ph 3 for where rates of acid and base catalyzed about equal.

Differs for exchanging hydrogens on other amide groups;

Minima:

- arg N, pH 2
- Lys N, pH 2.5
- Asn, Gln, pH 5
- Ser, Thr, pH 5.5
- Asp, Glu, pH 10

D. Hydrogen exchange within folded proteins

Even in unfolded chains, amide rates vary by more than 100-fold, due to neighboring group interactions.

Within folded proteins At least two sets:

- Conformation independent – e.g. surface arginine
- Conformation sensitive.
 - inductive effects through bonds electrostatic field effects, so large protein exchange behavior more complicated than sum of model compounds. For tight molecule, BPTI, in latter case slowest protons are 10^{-8} slower than model amides.

A rapidly exchanging, presumably flexible protein (insulin) has smaller range of exchange rates than inflexible BPTI.

Exchange may be:

- rapid: 0.1s, 10°C, pH 7.5
or
- slow: 1000s, 0°C, pH 3

In actual protein:

How to identify which individual groups are exchanging??

The identification of the exchangeable hydrogens depends on the development of **NMR spectroscopy**: (Creighton 2nd ed., 238-244)

nuclear spins; **only NMR signal from proteins is from hydrogens**;

However very sensitive to local electronic environment; both covalent and through space;

Can detect protein hydrogens if they are not rapidly exchanging with solvent;

Thus can detect non-exchangeable hydrogens' H bonded, buried in structure;

Walter Englander and colleagues:

Assigned complete resonances in both oxidized states of cytochrome c.

About **50 of 100 backbone amide protons exchange slowly enough in native state to locate by 2-D NMR.**

If little difference in rate of exchange in folded and unfolded, can't learn very much;

Valuable amides are those whose exchange rate is protected in the folded protein.

Two protocols:

- label exchangeable protons of unfolded form with tritium
- then dilute out of denaturant and into H₂O
- Will get competition between exchange out of tritium and refolding which traps tritium.
- Count radioactivity to get classes; NMR of product to detect individual residues H/H₃]

H/D exchange; H₂ does not give NMR peak: Pulse.

Time Scale of Hydrogen exchange; Roder and Wuthrich (1985) Biochemistry, **24**, 7407.
Wand, Roder and Englander (1986) Biochemistry 25, 1107.

From my notes

E. Direct Structural Characterization of a Kinetic Folding Intermediates

H. Roder, Gulner Elove, and S. Walter Englander (1988) "Structural characterization of folding intermediates in cytochrome c by H-exchange labeling and proton NMR" Nature, 335, 700-704.

[Note: Relevant figure may be found in H. Roder, Gulner Elove, and S. Walter Englander (1988) "Structural characterization of folding intermediates in cytochrome c by H-exchange labeling and proton NMR" Nature, 335, 700-704.]

General Experimental Strategy:

- Label all exchangeable hydrogens with deuterium in unfolded state:
- Allow exchange in with hydrogen during folding.
- Collect folded material, locate exchangeable Hs by 2-D NMR.

Requires protein that:

- is very soluble
- refolds at acid pH
- refolds in presence of residual denaturant

1. Unfold in denaturant/D₂O solution
 - a. deuterate all exchangeable NH sites
 - b. Start: 4.2M GdnHCl, D₂O, pH 6.0, 6mM protein, 10°C
2. Initiate refolding by dilution
 - a. First rapid dilution: 6-fold dilution to pH 6.2 in D₂O
3. Resulting 0.7M GuHCl is well below unfolding transition
4. Expose to pulse of water at high pH
 - a. At various intervals expose to 50ms pulse of glycine, sodium phosphate buffer in H₂O to pH 9.3
 - b. Under conditions chosen for pulse (pH 9.3, 10°C, 40-60ms), free peptide H-exchange time constant is about 1 ms, so that amide sites in still unstructured parts of protein become fully protonated.
 - c. On the other hand proton label is excluded from parts of protein that have already become structured.
5. quench H exchange with quench buffer, pH 5.3 at 0°C, which does not stop refolding
 - a. Terminate labeling pulse by further shift to slow exchange conditions, where refolding can continue terminate pulse after 50^{+/-}10 ms by injecting into
6. Now
 - a. concentrate protein
 - b. discard insoluble aggregated material
 - c. carry out NMR determination of buried proteins

Find the proton occupancies by 2-D NMR

Technically very difficult because it requires three rapid mixing stages. In addition - cannot take multiple time points from same sample:

For each **time of refolding** before pulse, from 4ms to 1second, need independent sample and independent experiment.

[Note: Relevant figure may be found in H. Roder, Gulner Elove, and S. Walter Englander (1988) "Structural characterization of folding intermediates in cytochrome c by H-exchange labeling and proton NMR" Nature, 335, 700-704.]

Here on the right is the refolding kinetics of cytochrome c at 10°C, monitored by fluorescence quenching of the single tryptophan 59.

Performed under similar conditions, **4.2M GuHCl > 0.7M, pH 6.0**, but at much lower protein concentration, 5x 10⁻⁶M.

The Time axis is logarithmic, and the fluorescence intensity is fraction of the fully unfolded. That is 1.0 is the value of the tryptophan fluorescence in the fully unfolded denatured state.

Three kinetic phases are resolved with relaxation times of 18 msec, 370 msec, 4 seconds.

Now let's examine the proton protection data:

The Y axis shows the proton occupancy; but remember experiment starts with proton deuterated; So if folds in flash proton occupancy will be zero! Or if give it long time to fold, deuterons will stay on, value will approach zero. But if only give it a short time to fold, then will get little protection, lots of exchange;

If all amide deuterons exchanged, proton occupancy at end of reaction would be 100%.

So early, before chain folded, get high value for exchange and for occupancy

If all amides stay deuterated, then proton occupancy at end of reaction would be zero:

Thus if right after diluting to refold, expose to protons, would get 100% exchange; If wait and let chain refold, some deuterons get protected, proton occupancy decreases with folding.

The X axis gives the time of refolding before exposure to a pulse.

The top curve shows the results for tryptophan 59. This is not a backbone amide but its indole Nitrogen hydrogen bonding to the heme group.

This high value means that exposure to hydrogens early in the folding exchanged almost all of them. The time of incubation needs to be long if the folding reaction was to provide protection.

The bottom curves are for the valine 11 and cysteine 14 amide protons. In this curve we see that protons were protected quicker, so seeing a more rapid folding transition.

[Note: Relevant figure may be found in H. Roder, Gulner Ellove, and S. Walter Englander (1988) "Structural characterization of folding intermediates in cytochrome c by H-exchange labeling and proton NMR" Nature, 335, 700-704.]

Note that the fluorescence behavior of the tryptophan 59 is faster than the tertiary H bond formation. Fluorescence phases look like N-terminal helix phases. Fluorescence drops by 50% while tryptophan 59 fully protonated.

So coming close to heme is not the same as forming H bond with heme propionate.

Similarly almost half of molecules forming H bond in slow phase, whereas fluorescence slow phase only 10% molecule folded quickly

[Note: Relevant figure may be found in H. Roder, Gulner Elove, and S. Walter Englander (1988) "Structural characterization of folding intermediates in cytochrome c by H-exchange labeling and proton NMR" Nature, 335, 700-704.]

Top curve leucine 64 and isoleucine 75
Bottom Curve L98 and Lysine 100 = C-terminal helix.

The degree of protection greatest for N and C-terminal helices: Occupancy drops to 40% in 30 msec.

Amide protons of 61 - 69 and 71 - 75 helices all behave like other phase.

The rapid protection of N- and C-terminal amide protons in a common kinetic phase distinct from all other protons suggest a special role for the terminal helices at an early stage of refolding.

In native cyt c two helices form tight contact near Gly 6 and Tyr 97.

Characteristic feature of all cytochromes:

- Early phase, 10 milliseconds,
- only terminal helices able to form stable structure to withstand proton labeling
- May be accompanied by some condensation but
- other helices not formed and
- most tertiary H-bonds still absent!
- A second kinetic phase of about 100msec, experienced by about half the molecules is characterized by H-bond formation throughout the molecules
- In a final 10 second phase, all remaining molecules acquire protection, native like molecules